(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
10 May 2002 (10.05.2002)

PCT

(10) International Publication Number
WO 02/36818 A2

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/GB01/04891

(22) International Filing Date:
2 November 2001 (02.11.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0026834.2 2 November 2000 (02.11.2000) GB

(71) Applicant *(for all designated States except US)*: **CONSEJO SUPERIOR DE INVESTIGACIONES CIENTIFICAS** [ES/ES]; Serrano, 117, E-28006 Madrid (ES).

(71) Applicant *(for SD only)*: **RUFFLES, Graham, Keith** [GB/GB]; 57-60 Lincoln's Inn Fields, London WC2A 3LS (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **MARTIN, Verónica** [ES/ES]; Gardenia, 2, Madrid (ES). **CARRILLO, Graciela** [ES/ES]; Callejón de los Morales, 19, Manzanares el Real, E-28410 Madrid (ES). **GUERRERO, Isabel** [ES/ES]; Calle Urano, 46, Tres Cantos, E-28760 Madrid (ES). **TORROJA, Carlos** [ES/ES]; Calle Lagasca, 129, E-28006 Madrid (ES).

(74) Agent: **RUFFLES, Graham, Keith**; Marks & Clerk, 57-60 Lincoln's Inn Fields, London WC2A 3LS (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/36818 A2

(54) Title: METHOD FOR DETECTING INHIBITORS OF TUMOUR GROWTH

(57) Abstract: Compounds that block HH signalling through modifications of PTC and SMO vesicular sorting are of use in the preparation of medicaments for the treatment of a mammalian cancer.

METHOD FOR DETECTING INHIBITORS OF TUMOR GROWTH

FIELD OF THE INVENTION

The present invention relates to a method of detecting inhibitors of tumor growth.

BACKGROUND OF THE INVENTION

The *Hedgehog* (HH) family of signalling molecules function as organisers in many morphogenetic processes. A surprising number of apparently unrelated human diseases, including familial and sporadic cancers and a number of syndromes and malformations, seem to be associated with abnormal function of the HH signalling pathway.

The gene *Patched* was found, in studies on development of the fruitfly drosophila, to function in relaying signals in the *Hedgehog* pathway. In the fly this signalling pathway is critical for proper embryonic development, and in vertebrates.

The role of HH signalling and its transduction pathway were first defined by observing its effects on the patterning of the embryonic segments of *Drosophila*. HH protein universally specifies and regulates the growth and differentiation of organ systems in eukariotes. We now know that HH also plays key roles in numerous other aspects of embryonic, larval and adult development in the fly.

In vertebrates there are at least three homologues, Sonic, Desert, and Indian, which play important roles in the development of many cell types and organs including the brain, bone, skin, gonads and lungs. The HH proteins are secreted and are thought to function as morphogens, signals that elicit concentration dependent responses from target cells. To fulfill these roles, both the distribution of HH molecules and the response of cells to HH must be tightly regulated. Clues to the mechanism that moves HH to its target cells have also come from the genetic analysis in *Drosophila*.

Genetic, biochemical and molecular analyses in both insects and vertebrates have resulted in a model for the intracellular transduction of HH signals from the membrane to the nucleus which has been conserved for over 300 million years. In contrast to most signalling pathways, intracellular HH signal transduction proceeds largely by sequential repressive interactions. The canonical pathway requires the activity of two transmembrane proteins *Patched* (PTC) and *Smothened* (SMO). In the absence of ligand, PTC downregulates the activity of SMO. The inhibition of this downregulation is released upon HH binding, activating the signal transduction cascade that leads to the function of Cubitus interruptus protein (GLI proteins in mammals).

Certain mutations in the human *Patched* gene which lead to excess activity of the *Hedgehog* pathway give rise to Basal Cell carcinomas, a type of skin cancer. It is thought that other alterations in the function of this pathway may lead to other types of mammalian cancers, especially skin cancers. Mutations that inactivate the pathway give rise to defects in development in vertebrates. Compounds have been found which can inactivate the pathway leading to malformations when acting on mammalian embryos, but causing no damage in adults.

In addition to HHs essential roles in normal development, a deregulated HH pathway is responsible for several human diseases, syndromes and

malformations. Holoprosencephaly (HPE), caused by a haploinsufficiency of *Sonic Hedgheog (SHH),* is a common dominant syndrome affecting the head and face. Loss of *Shh* function in mice similarly leads to HPE. HPE was also found in farm animals exposed to cyclopamine, a plant alkaloid that interferes with cholesterol's role in SHH signaling. Similarly, Smith-Laemli-Opitz syndrome patients are deficient in cholesterol biosynthesis and can display HPE traits.

In contrast to loss of SHH signaling in HPE, there are several types of cancers that may be caused by inappropriate SHH signaling. Sporadic basal-cell carcinoma (BCC) is the most common type of skin cancer, with a high incidence in elderly Caucasians. Nearly all BCCs show expression of *Gli1* (one of the trascriptional modulators of the HH pathway) which may be responsible for BCC formation, and may be induced by a number of mutations, including those that inactivate PTCH or activate SMO. Familial BCCs in basal cell nevus syndrome (BCNS; or Gorlin's syndrome) patients also derive from loss of PTC function. Two other sporadic cancers, also seen in BCNS patients, are similarly due to altered SHH pathway signaling. Both Medulloblastoma, a rare but deadly childhood primitive neuroectodermal tumor of the cerebellum, and rhabdomyosarcoma, a muscle cancer, may be derived from mutations in PTCH.

Compounds able to inactivate the *Hedgehog* pathway are potentially useful for the reversal of tumorigenesis leading to basal cell carcinoma.

SUMMARY OF THE INVENTION

Data are presented here which indicate that the sterol-sensing domain of *Patched* protein is required for *Hedgehog* signalling but not for *Hedgehog* sequestration.

Therefore, we predict that compounds which bind to this domain are potential

inhibitors of *Hedgehog* signalling and therefore useful to revert proliferation of mammalian cancers, especially skin cancers, and in particular basal cell carcinomas. Such compounds may be, but are not limited to, analogues and derivatives of sterols such as cholesterol.

Furthermore, we predict that compounds that block HH signalling through modifications of PTC and SMO vesicular sorting will be useful to revert proliferation of mammalian cancers, especially skin cancers, and in particular basal cell carcinomas.

The compounds described in the foregoing two paragraphs (hereinafter referred to a "compounds of the invention") may be detected by an assay system where a reporter gene (for example firefly luciferase) is placed under the control of a promoter dependent on *Hedgehog* signalling for expression in a mammalian cell culture system. Treatment of the cells with compounds inhibiting the patch sterol sensing domain or other proteins functioning in this signalling system, will change the expression level which can be detected by measuring the product of the reporter gene (in the case of firefly luciferase, by chemoluminescence).

The present invention provides the compounds of the kind which can be detected by the assay, as well as pharmaceutical compositions, methods of treatment and related embodiments.

Thus, the present invention provides a method of treating any mammal, notably a human, affected by cancer which comprises administering to the affected individual a therapeutically effective amount of a compound of the invention, or a pharmaceutical composition thereof.

The present invention also relates to pharmaceutical preparations, which contain as active ingredient a compound or compounds of the invention, as well as the

processes for their preparation.

Examples of pharmaceutical compositions include any solid (tablets, pills, capsules, granules, etc.) or liquid (solutions, suspensions or emulsions) with suitable composition or oral, topical or parenteral administration, and they may contain the pure compound or in combination with any carrier or other pharmacologically active compounds. These compositions may need to be sterile when administered parenterally.

Administration of the compounds or compositions of the present invention may be by any suitable method, such as intravenous infusion, oral preparations, intraperitoneal and intravenous administration. We prefer that infusion times of up to 24 hours are used, more preferably 2-12 hours, with 2-6 hours most preferred. Short infusion times which allow treatment to be carried out without an overnight stay in hospital are especially desirable. However, infusion may be 12 to 24 hours or even longer if required. Infusion may be carried out at suitable intervals of say 2 to 4 weeks. Pharmaceutical compositions containing compounds of the invention may be delivered by liposome or nanosphere encapsulation, in sustained release formulations or by other standard delivery means.

The correct dosage of the compounds will vary according to the particular formulation, the mode of application, and the particular situs, host and tumour being treated. Other factors like age, body weight, sex, diet, time of administration, rate of excretion, condition of the host, drug combinations, reaction sensitivities and severity of the disease shall be taken into account. Administration can be carried out continuously or periodically within the maximum tolerated dose.

The compounds and compositions of this invention may be used with other drugs

to provide a combination therapy. The other drugs may form part of the same composition, or be provided as a separate composition for administration at the same time or a different time. The identity of the other drug is not particularly limited, and suitable candidates include:

a) drugs with antimitotic effects, especially those which target cytoskeletal elements, including microtubule modulators such as taxane drugs (such as taxol, paclitaxel, taxotere, docetaxel), podophylotoxins or ymca alkaloids (vincristine, vinblastine);

b) antimetabolite drugs such as 5-fluorouracil, cytarabine, gemcitabine, purine analogues such as pentostatin, methotrexate);

c) alkylating agents such as nitrogen mustards (such as cyclophosphamide or ifosphamide);

d) drugs which target DNA such as the antracycline drugs adriamycin, doxorubicin, pharmorubicin or epirubicin;

e) drugs which target topoisomerases such as etoposide;

f) hormones and hormone agonists or antagonists such as estrogens, antiestrogens (tamoxifen and related compounds) and androgens, flutamide, leuprorelin, goserelin, cyprotrone or octreotide;

g) drugs which target signal transduction in tumour cells including antibody derivatives such as herceptin;

h) alkylating drugs such as platinum drugs (cis-platin, carbonplatin, oxaliplatin, paraplatin) or nitrosoureas;

i) drugs potentially affecting metastasis of tumours such as matrix metalloproteinase inhibitors;

j) gene therapy and antisense agents;

k) antibody therapeutics;

l) other bioactive compounds of marine origin, notably kahalalide F, the ecteinascidins such as ecteinascidin 743, or the didemnins such as aplidine;

m) steroid analogues, in particular dexamethasone;

n) anti-inflammatory drugs, in particular dexamethasone; and

o) anti-emetic drugs, in particular dexamethasone.

The present invention also extends to the compounds of the invention for use in a method of treatment, and to the use of the compounds in the preparation of a composition for treatment of cancer.

In summary therefore, the *Hedgehog* (Hh) family of signaling molecules function as organizers in a wide variety of morphogenetic processes, in vertebrates and invertebrates. Morphogenesis in *Drosophila* has been widely used as a model system to examine Hh function. Accumulated data ascribe an important role to cholesterol in Hh signalling, both in signal generation and reception. *Patched* (Ptc), an Hh reception complex protein, has a negative role in Hh signalling and is able to block the pathway and control Hh diffusion. Five of the twelve transmembrane segments of Ptc protein are homologous to the conserved region, the sterol-sensing domain (SSD), originally identified in several proteins involved in cholesterol homeostasis. The present inventors have conducted a study in which the functional role of the SSD of Ptc was addressed in flies. Ptc protein with a single aminoacid substitution in the SSD, shows dominant negative activity for *ptc* in inducing target gene activation in a ligand-independent manner. This mutated form of Ptc, however, sequesters and internalises Hh as does the wild type protein. This aminoacid change was also identified in a preexisting *ptc* allele.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1

Figure 1A shows that ectopic Ptc^{SSD} expression induces the embryonic *ptc* mutant phenotype. Figure 1A shows the cuticle phenotype of wild type (a), *UAS-ptc^{SSD} /ptc-GAL4* (b), and *ptc^{IIW109}* (c) larvae. Note that the involution of

the head is not well formed (asterisk) and the thoracic and first abdominal segments are absent in *ptc*⁻ larvae (arrows) (c). In *UAS-ptc*^{SSD} */ptc-GAL4* these structures are still present (arrows) but the rest of abdominal segments are as in *ptc*⁻ embryos (b). Wg expression in the wild type (d) is restricted to single cell stripes and in *UAS-ptc*^{SSD} */ptc-GAL4* embryos (e), Wg stripes are wider than in wild type embryos, as observed in *ptc*⁻ embryos (data not shown).

Figure 1B shows that ectopic Ptc^{SSD} expression in the wing primordia induces the activation of Hh target genes.

a, b, c. Wild type expression of *dpp (a), ptc* (b), and *caupolican* gene of the *iro* complex (c) in the third instar wing imaginal disc.

Ectopic *ptc*^{SSD} clones (green) induce *dpp* (red) (d) and *caupolican* expression (green) (e) in the A compartment of wing imaginal disc. Uniform Ptc^{SSD} expression using *765-GAL4* line induces the expression of the endogenous *ptc* (shown in red by the *ptc-LacZ* reporter gene expression) and homogenous high leves of citoplasmic Ci (green) (1).

Figure 1C shows that Ectopic Ptc^{SSD} expression in the wing primordia induces ptc⁻ wing phenotype.

This ptc^{SSD} activation give rises to overgrowth and vein desorganization of the A compartment in *UAS-ptc*^{SSD}*/ 765-GAL4* adult wing (a) compared to wild type wing (b). Ectopic Ptc^{WT} expression using *765-GAL4* line gives the same phenotype as the ectopic Ptc^{F} that contains the other mutation found in the ptc^{S2} allele (Val^{1392} to Met substitution). (c). Note the wing size reduction produced by blocking the induction of the Hh targets at the A/P compartment border and compare with the overgrown *UAS-ptc*^{SSD}*/ 765-GAL4* wings shown in (e).

Figure 2

Figure 2A shows Hh sequestration by Ptc^{SSD}.

a, b, c, and d. Hh (green) is sequestered and internalised by Ptc (red). This occurs in both Ptc^{WT} (a) and Ptc^{SSD} ectopic posterior clones. (c). Detail of these Ptc^{WT} (b) and Ptc^{SSD} (d) clones. Note the higher levels of Hh in Ptc expressing cells and also the co-localisation of Ptc and ITh proteins in the same punctate vesicular structures.

Figure 2B shows that Ptc^{SSD} blocks the induction of the Hh target genes at the A/P border.

a, Wild type *engrailed* (red) and Ci (green) expression.

b. Ptc^{SSD} expressed in the *engrailed* domain *(UAS-*Ptc^{SSD}*/ en-GAL4)* decreases the activation of the target genes at the A/P compartment border, such as the late *en* expression in the A compartment and the cytoplasmic accumulation of Ci. Comparison with the wild type expression pattern of these genes (a). c, wild type *dpp* expression (red). d. *dpp* expression is reduced in *UAS-*Ptc^{SSD}*/ en-GAL4* imaginal discs. e. Phenotype of *UAS-*Ptc^{SSD}*/ en-GAL4* adult wings. A similar phenotype is observed in *UAS-*Ptc^{WT}*/ en-GAL4* wings (data not shown).

Figure 3

Figure 3A shows that ectopic Smo elicits similar cellular responses and phenotype as Ptc^{SSD}. a, Ectopic Smo clones (red) induce high levels of Ptc expression (green) in the A compartment. b, Uniform high levels of Smo protein (green) driven by the 765-GAL4 line activates *dpp* (red) in the A compartment of the wing disc. However, genes that respond to maximum values of Hh such as *engrailed* expression in the A compartment and *collier* are not activated (data not shown). Note that the responses to either ectopic Smo and PtcSSD are the same. c, Phenotype of *UAS-Smo/765-GAL4* wings. Note that the overgrowth and vein alteration of the A compartment of the wing is similar to that observed in *UAS-*Ptc^{SSD}*/ 765-GAL4* wings shown in Fig. 1.

Figure 3B shows that Ptc^{SSD} causes the stabilisation of Smo levels.

a. Smo staining pattern (green) and Ptc (red) in wild type imaginal discs. Note the higher Smo protein levels in the A/P compartment border and in the P compartment. b. Ectopic Ptc^{SSD} clones induced in the wing imaginal disc and marked by the lack of CD2 staining (red). c. Ectopic Ptc^{WT} clones marked by the presence of Ptc protein (red). Note that Ptc^{SSD} but not in Ptc^{WT} clones autonomous increase of Smo levels in the A compartment. Note also that ectopic Ptc^{WD} but Ptc^{SSD} clones negatively modulate Smo levels in the P compartment.

Figure 4

Figure 4 shows the HH diffusion through a *ptc*^{IIR87} (L83Q) mutant clone. The clone was induced in a third instar wing imaginal disc. The disc was stained with anit-PTC (green) and anti-β gal (red) antibodies. The mutant clone, marked by the lack of anti-β gal staining (red), was located in the anterior compartment abutting the anterior / posterior compartment border (outlined in white). PTC expression inside and outside the clone revealed the increase of HH signal diffusion trough the clone (dotted line). Note that HH produced in the posterior compartment (that did not express PTC), travels through the clone and signals inside (outlined in white) and outside the clone (dotted line). It also shows the different sub-cellular localisation of PTC protein inside the clone compared with the surrounded wild type cells (vesicular cellular structures or dotts are indicated by arrowhead).

RESULTS AND DISCUSSION

Hh signal reception requires the activity of two transmembrane proteins Patched (Ptc)

and Smothened Smoothened (Smo) (reviewed in Ingham, 1998). Genetic evidence indicates that Ptc is a component of the Hh reception complex and biochemical findings suggest physical interaction between the vertebrate

homologue of Ptc and Sonic hedgehog (Shh) (Mango et al., 1996). Ptc contains 12 predicted trans-membrane domains with two large extra-cellular loops and two internal amino and carboxyl terminal tails (Hooper and Scott, 1989; Nakano et al., 1989). Smo, the other component of the Hh reception complex, is a seven trans-membrane domain protein reminiscent of G-protein-coupled receptors. Hh signalling is fully mediated by Smo, since lack of Smo precludes cells from responding to Hh. In the absence of ligand, Ptc inhibits the activity of Smo; the inhibition is released upon Hh binding, activating the signal transduction cascade (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). The mechanism by which Ptc regulates Smo is not well understood, although recent findings suggest that Ptc downregulates Smo protein levels (Alcedo et al., 2000; Denefet al., 2000).

In Hh signalling, cholesterol is important for ligand biogenesis, release of Hh from producing cells and for signal transduction in receiving cells, having perhaps different roles at multiple points (Review in Icardona and Eaton, 2000). The SSD was originally identified in two proteins: HMGCoA reductase and SREBP (sterol regulatory element binding protein) cleavage activating protein (SCAP). In addition, Ptc is structurally similar to the Niemann-Pick Cl (NPC1) protein that participates in intracellular cholesterol transport (Loftus et al., 1997; Carstea et al., 1997). The functional role of the SSD of Ptc has been addressed by site directed mutagenesis. To choose a specific mutation, we considered a recurrent G-to-A substitution in a single codon (Asp^{443}-to-Asn) in the SSD of SCAP protein that causes sterol-resistance in mutant Chinese hamster ovary cell lines (Nohturfft et al., 1996). This Asp^{443} is conserved in the SSD of all six family members in mouse, human and *C. elegans* NPC1 proteins (Loftus et al., 1997) and is also present in Ptc. Thus, we induced a point mutation, G to A, at position 1749 of *ptc* c-DNA to produce the aminoacid change from Asp^{584} to Asn in the SSD of Ptc protein. The mutated cDNA was introduced in flies under UAS control sequences. The mutant form of the protein was over-expressed in

its own expression domain using the *ptc-GAL4* line. The phenotype of Ptc^{SSD} overexpression was examined in both embryonic and larval development. The Ptc^{SSD} protein competed with the endogenous wild type Ptc protein resulting in embryonic lethality and in an almost complete *ptc* null mutant phenotype (Fig. 1A, panels a,b and c). This competition between the two Ptc proteins was confirmed by the reduction of the *ptc* phenotype in conditions of lower induction of the mutant form of the protein (data not shown).

An essential process in pattern formation in flies is the setting of two adjacent populations of cells: the posterior (P) and anterior (A) compartment cells (Garcia-Bellido et al., 1973), or those that express *hedgehog* (P cells) and those that respond to the Hh signal (A cells) (reviewed in Lawrence and Struhl, 1996). At the A/P compartment border, Hh induces the expression of other morphogenetic signal molecules such as *wingless (wg)* and the TGF-beta super-family member, *decapentaplegic* (dpp) (reviewed in Lawrence and Struhl, 1996). Since Ptc is a repressor of *wg* expression in the embryo, a characteristic of *ptc* mutants is the extension of *wg* expression in the embryonic epidermis towards the anterior part of the segment. Flies expressing the UAS-Ptc^{SSD} showed an extension of the *wg* stripe in each embryonic segment (Fig. 1A, e).

In wild type wing imaginal discs, particular Hh levels elicit different gene responses at the A/P border (Mullor and Guerrero, 2000). A characteristic feature of homozygous ptc^- clones in the A compartment of the wing imaginal discs is the autonomous activation of all Hh target genes in the absence of ligand. We therefore evaluated whether the Ptc^{SSD} protein was able to activate Hh pathway targets in adult primordia. Ectopic UAS-Ptc^{SSD} clones autonomously activated target genes in the A compartment that respond to lower Hh levels such as *dpp* (Fig. 1 B, a), the *iroquois* complex genes (Fig. 1 B, b) and the endogenous *ptc* itself (Fig. 1B, c). However, genes that respond to maximum values of Hh such as the late *engrailed (en)* expression (Ohlmeyer and Kalderon,

1998) and *collier* (Nestoras et al., 1997; Vervoort et al., 1999) were not activated (data not shown). The endogenous wild type Ptc protein might compete with the mutant protein permitting activation of the target genes that respond to lower Hh levels. Thus, the SSD mutated form of Ptc protein has a dominant negative function that interferes with the ability of endogenous Ptc to inhibit target gene transcription, provoking a *ptc*⁻ wing phenotype (Fig. 1C, b). These results suggest that cholesterol's role in the Hh pathway is to control Ptc function.

We then determined if the other function of Ptc, Hh sequestration (Chen and Struhl, 1996), was modified in Ptc^{SSD}. It is known that *ptc* transcription in anterior cells is up-regulated in response to Hh signalling (Nakano et al., 1989; Capdevila et al., 1994; Tabata and Kornberg, 1994) and that these high levels of Ptc protein limit the range of Hh signalling (Chen and Struhl, 1996). The co-localisation of Hh and Ptc proteins in vesicular punctate structures in the Hh-receiving cells has also been observed, suggesting that Ptc internalises Hh to control Hh diffusion in A/P compartment border cells (Tabata and Kornberg, 1994; Ramirez-Weber, 2000). We noted this effect of Ptc sequestering Hh in clones induced in the posterior compartment, where Ptc is not normally present (Fig. 2A, a and b). Hh staining was diffuse in wild type P compartment cells. However, the Hh staining pattern changed in Ptc expressing clones, where both Ptc and Hh co-localised in the same punctate structures (Fig. 2A, b). Furthermore, Hh protein levels were higher in Ptc expressing clones than in the rest of the P compartment (Fig. 2A, a). Ectopic Ptc^{SSD} clones in the P compartment provoked the same changes in the Hh staining pattern as Ptc^{WT} protein (Fig. 2A, c and d). These findings suggest that ptc^{SSD} protein was able to sequester and internalise Hh as the wild type protein.

In addition, ectopic Ptc^{WT} in the entire P compartment using the *en-GAL4* driver is able to restrain the secretion of Hh at the A compartment, impeding the appropriate formation of the Hh gradient and the correct activation of the target

genes (Johnston et al., 1995). Ectopic Ptc^{SSD} or Ptc^{WT} driven by the *en-GAL4* line partially blocks the response to Hh at the A/P compartment border, as shown by the decrease of *dpp* expression, the lack of late *en* expression in the A compartment and the absence of increased cytoplasmic levels of the nuclear efector of the Hh pathway, Cubitus interruptus (Ci) (Fig. 2B). This low Hh response at the A/P border causes fusion of wing veins 3 and 4 (Mullor et al., 1997) (Fig. 2B, e). Taken together, these results demonstrate that the SSD of Ptc is not necessary for Hh sequestration.

A complementation assay was performed on *ptc* alleles with a dominant gain of function allele of *ptc*, ptc^{Con} (Mullor and Guerrero, 2000), to search for mutations in different Ptc functional domains. ptc^{Con}/ptc^{-} flies died at third larval instar and the imaginal discs formed were small because most of the Hh targets are not activated. Genetic interaction between Ptc^{Con} and Hh and the localisation of a point mutation in one of the extracelullar loops of Ptc suggests that this Ptc^{Con} protein affects Hh-binding (Mullor and Guerrero, 2000). Two out of 15 *ptc* alleles, ptc^{S2} and ptc^{Tuf2}, were found to complement with ptc^{Con}. Thus, we sequenced these alleles and found two point mutations in identical positions indicating that both alleles were originally the same despite the difference in nomenclature. The first mutation was located in the SSD (base 1749 is mutated from G to A) giving an Asp^{583}-to-Asn substitution and the second mutation was located in the carboxy-tail of the protein (base 4174 is mutated from G to A) giving a Val^{1392} to Met substitution. The first point mutation was identical to the Ptc^{SSD} mutation. To test if the second point mutation of ptc^{S2} could be also responsible for the ptc^{-} phenotype of this allele, a cDNA containing this second point mutation was introduced in flies under UAS control. The flies expressing this mutant Ptc protein were viable and showed a phenotype similar to the one produced by overexpressing the wild type protein (Fig. 1C, e). Therefore, we concluded that the mutation in the SSD is responsible for the lack of function phenotype in the ptc^{S2} allele. This result agrees with previous work

demonstrating that the *ptc*^{S} allele behaved as a lack of function allele of *ptc* for blocking the Hh pathway but the mutant protein produced was still able to bind Hh (Chen and Struhl, 1996).

The ability of some proteins to preferentially associate with membrane sub-domains rich in cholesterol and sphingolipids, called rafts, is thought to be the basis for specific clustering events that regulate membrane trafficking and signal transduction (Simons and Ikonen, 1997). Since raft membranes are rich in cholesterol, it is possible that cholesterol linkage might be a raft-targeting signal. Recently, it has been found that Hh modified by cholesterol is associated with rafts (Rietveld et al., 1999). Although, Ptc association with rafts is not known, it might be required for Hh binding. Ptc might require sterol modification or interaction for its association with rafts. However, our results indicate that, regardless of whether Ptc is localised in the rafts or not, the mutation in the SSD does not affect the sequestering of Hh. Thus, it is possible that the mutated Ptc^{SSD} still preferentially associates with rafts but fails to interact with other components of the signal transduction machinery such as Smo.

The balance between Ptc and Smo proteins seems to be fundamental in blocking or activating the Hh pathway (Denef et al., 2000; Alcedo et al., 2000). In wild type wing imaginal discs, *Patched* downregulates Smo protein levels in the absence of Hh. Smo levels are high in the P compartment and in Hh-responsive A cells. Smo expression decreases sharply near the boundary and then more gradually across the A compartment (Denef et al., 2000; and Fig). We found that the overexpression of Smo induced the activation of the Hh pathway, suggesting that part of the Smo protein cannot be downregulated by Ptc. Thus, ectopic Smo expression produces the same activation of the target genes (Fig. 3A, a and b) and the same phenotype (Fig. 3A, c) as ectopic ptc^{SSD}. Consistently, this phenotype was reversed when both wild type Ptc and Smo proteins were co-expressed (data not shown). To analyse how Smo protein

levels are modulated by Ptc^{SSD} compared with the Ptc^{WT} protein, we examined the Smo staining pattern in ectopic clones of these two Ptc protein forms. Ectopic Ptc^{SSD} but not Ptc^{WT} clones increased Smo levels in the A compartment (Fig. 3B arrows). Ectopic Ptc^{WT} but not Ptc^{SSD} clones negatively modulated Smo levels in the P compartment (Fig. 3B arrowheads). These results indicate that Ptc is a direct modulator of Smo levels according to the results recently reported by Denef et al., 2000. The opposite modulation of Smo levels in cells that ectopically express either Ptc^{SSD} or Ptc^{WT} proteins indicates that Ptc^{SSD} causes the stabilisation of Smo levels, probably because it cannot interact with Smo. This negative and positive regulation of Ptc and Smo in Hh signalling has been described in tumorogenic processes in vertebrates (review in Goodrich and Scott, 1998) where Ptc acts as tumour suppressor gene by repressing the oncogenic activity of Smo during transduction of the Hh signal (Hahn et al., 1996; Johnson et al., 1996; Xie et al., 1998; Taipale et al., 2000).

It has recently been described that oncogenic mutations in Smo and Ptc can be reversed by inhibitors of cholesterol synthesis such as cyclopamine. These steroidal alkaloids appear to be specific antagonists of Shh signal transduction in mouse and chick and produces a phenocopy of an Shh loss-of-function mutation (Cooper et al., 1998; Incardona et al., 1998). Cyclopamine inhibits the Shh pathway by antagonising Smo and may act by affecting the balance between active and inactive forms of Smo (Taipale et al., 2000). Herein, we show that mutations in the SSD of Ptc exert the opposite effect to Ptc^{WT} giving rise to higher Smo activity. This effect is similar to that produced by increasing Smo protein levels. Like cyclopamine, Ptc activity shifts the Smo balance towards the inactive state (Taipale et al., 2000 and the present study). These results all indicate that the SSD domain of Ptc is required to negatively regulate Smo activity.

A similar molecular mechanism for Ptc and NPC1 function has been suggested

since both Shh signalling and NPC1-mediated cholesterol transport are inhibited by the same steroidal components (reviewed in Incardona and Eaton, 2000). Although the precise function of NPC1 is unknown, the protein is normally found in cytoplasmic vesicles that are relatively cholesterol poor and show the characteristics of late endosomes (Neufeld et al., 1999). It has been suggested that a lesion in the NPC1 protein produces a general defect in the retrieval and recycling of raft components of the endocytic pathway (Blanchette-Mackie et al., 2000; Kobayashi et al., 1999). Interestingly, we previously reported that Ptc is located inside the cell, mainly in cytoplasmic vesicles and that when internalisation and trafficking are blocked in *shibire* mutant embryos, Ptc is associated with the plasma membrane (Capdevila et al., 1994). The genetic evidence presented here suggests that in the Hh-receiving cells, cholesterol modulation is required - not for Hh binding to Ptc - but for the signalling activity of Ptc via its sterol sensing domain. Cholesterol modulation would allow Ptc to interact with Smo or with Hh in distinct intracellular compartments. Accordingly, Denef et al., 2000 propose distinct subcellular localisations of Ptc and Smo in Hh-stimulated cells. Although the role of cholesterol in the Ptc-mediated trafficking process through endocytic compartments is still unclear, we propose that in ptc^{SSD} mutants, Ptc cannot block the activity of Smo due to a failure in vesicular sorting.

Thus, in the above, we have suggested that PTC has at least two functions; one involved in signaling through an interaction with Smo and the other implicated in sequestering HH. We have found two mutations in Ptc protein that complement each other. These lesions reveal two functional domains of PTC that control HH signaling. One domain is important in regulating the interaction with second messengers such as SMO while the other domain is involved in controlling the morphogenetic gradient of HH. Altogether our results indicate that these two domains of PTC protein control HH signalling through vesicular trafficking.

The Sterol Sensing Domain mutant protein (D584N) causes an accumulation of PTC in the endocitic compartment. This accumulation of PTC prevents the interaction and further degradation of the receptor molecule SMO. This functional alteration of PTC provokes the constitutive opening of the HH pathway. However, PTC function in sequestering HH remains normal.

We have undertaken a further study, whose results show that a mutation (L83Q) in the other PTC domain prevents the sequestration and further degradation of HH. However, the interaction with Smo is normal. PTC blocks SMO and only opens the HH pathway when HH is present. The HH gradient is expanded in PTC (L83Q) mutant cells because the internalized HH cannot be degraded and is released again to the extracellular matrix (See Figure 4). These investigations demonstrate that PTC regulates the recycling and degradation of HH to establish the formation of its morphogenic gradient.

Previous observations indicate that steroidal alkaloids analogous to cholesterol such as Cyclopamine, can reverse oncogenic mutations in SMO and PTC. This steroidal alkaloid appears to be a specific agonist of SHH signal transduction in mouse and chick and produces a phenocopy of the SHH loss-of-function mutation. Cyclopamine inhibits the SHH pathway by antagonising SMO and may act by affecting the balance between active and inactive forms of SMO. Like Cyclopamine, Ptc activity shifts the SMO balance toward the inactive state.

Based on the results presented, our hypothesis is that Cyclopamine might modify the subcelular localisation of Ptc and/or Smo. Our data suggest that Ptc and Smo could be the direct or indirect targets of Cyclopamine causing a block in the Hh signaling pathway. These findings are related to the mechanism of how the putative antitumoral molecule Cyclopamine is acting to block the HH pathway.

The discovery of two Ptc functional domains related to vesicular sorting allows

screening for compounds that can modify the Hh, Ptc and Smo vesicular trafficking in mammalian cells. This provides a method to detect inhibitors of tumour growth.

MATERIALS AND METHODS

Fly stocks

The FlyBase (http://gin.ebi.ac.uk:7081) entries of the mutations, insertions and transgenes as referred to in the text are as follows:

ptc⁻: ptc^{16} or ptc^{IIw109} , an amorphic allele of the *ptc* gene.

ptc^{S2} : ptc^{S2} , embryonic lethal allele of the *ptc* gene.

FRT42: P {ry[+t7.2]=neoFRT}42D

hsp7O-flipase (FLP122)

The reporter genes *dpp-LacZ* (BS 3.0) and *ptc-LacZ* are expressed as their endogenous RNAs in the imaginal disc (Blackman et al., 1991). For the ectopic expression experiments using the GAL4/UAS system (Brand and Perrimon, 1993), the following GAL4 drivers were used: c765-GAL4 (ubiquitously expressed in the wing disc (Brand and Perrimon, 1993); *ptc*-GAL4 (Hinz et al., 1994), en-GAL4 (a gift from A. Brown); *hh*-GAL4 (a gift from T. Tabata). The UAS-*smo* driver was constructed using full length *smo* cDNA (Mullor and Guerrero, 2000). Several UAS-*smo* fly lines were obtained but only some of them yielded phenotypes with the GAL4 lines tested.

Genotypes of larvae for generating mosaic clones.

Clones were generated by FLIP-mediated mitotic recombination (Golic, 1991; Xu and Rubin, 1993). FLP-mediated recombination was induced by incubating larvae of the following genotypes at 37^0C for 15 minutes 48-96 hours AEL.

Mutant clones

hs.FLP; FRT42D ptc^{S2}/FRT42D arm-Lac-Z

hs.FLP; FRT42D ptc^{S2}/FRT42D Myc; Dpp-Lac-Z

Flip-Out clones

To generate random clones of wild type ptc, ptc^{SSD} mutation and Smo, a hybrid of the Flip-out and GAL-4 activation systems, was used. Clones expressing GAL-4 were induced by flipping out an interruption cassette from Actin>CD2>GAL-4 (Pignoni and Zipursky, 1997) or abx>Bgal>GAL4 (de Celis and Bray, 1997) transgenes in a genetic background containing the corresponding UAS-cDNA, dpp-Lac-Z. The clones were induced by incubating larvae of the corresponding genotypes at 37^{0}C for 15 minutes.

Ptc^{WT}: Act>CD2> GAL4 / HSFLP122; UAS-Ptc^{WT};

Ptc^{SSD}: Act>CD2> GAL4 / HSFLP122; UAS-Ptc^{SSD} / dpp-LacZ;

Smo: Act> CD2>GAL4 / HSFLP122; UAS-Smo / dpp-LacZ

Smo: abx>Bgal>GAL4 / HSFLP122; UAS-Smo /dpp-LacZ

To modify the levels of the UAS construct, we took advantage of the temperature sensitivity of the GAL4 system (Wilder and Perrimon, 1993). Using the same GAL4 line, the effects of different levels of the protein at set temperatures were compared.

In vitro induction of point mutations

The QuickChange™ Site-Directed Mutagenesis Kit from Stratagene was used to introduce point mutations. These induced mutations were subsequently confirmed by sequence analysis. Wild type ptc cDNA and the mutant cDNA forms were introduced in the UAS vector that contains the sequences responding to the yeast GAL4 protein. Several independent lines were obtained. We also

obtained new UAS-Ptc^{WT} lines that produced different levels of Ptc protein. All experiments involving the three Ptc protein forms (wild type and mutant forms) were conducted using only the transgenic fly line generated that showed equivalent levels of Ptc protein product after induction with GAL4.

Immunostaining of imaginal discs.

Immunofluorescence staining was performed as described in Sánchez-Herrero et al., 1996. Antibodies were used at the following dilutions: rat polyclonal anti-Smo (Denef et al., 2000) 1/200; rabbit polyclonal anti-Col antibody (Vervoort et al., 1999) 1/200; mouse monoclonal anti-En (Patel et al., 1989) 1/10; rat monoclonal anti-Ci, which recognises the C-terminal end of the protein and thus the un-proteolised form of Ci (Motzny and Holmgren, 1995) 1/10; rat polyclonal anti-Caupolican. This antibody cross-reacted with Araucan but not with Mirror (Diez del Corral et al., 1999) 1/1000; mouse monoclonal anti-Ptc (Apa 1.3) (Capdevila et al., 1994) 1/100; rat polyclonal anti-Hh (Sanchez-Herrero et al., 1996) 1/100; rabbit polyclonal anti-β-gal (from Jackson laboratories) 1/1000; mouse monoclonal anti-Myc 9E10 (from BabCO, Berkeley antibody company) 1/1000. Stained imaginal discs were examined under a Zeiss Confocal Laser Scan microscope.

Characterisation of the *plc* S2 allele.

DNA from ptc^{S2}, *ptc^{tuf2}* and *cn bw sp* larvae was extracted following standard protocols. Small fragments of *ptc* covering all *ptc* coding sequences were amplified by PCR and sequenced using the ABI PRISM^{TM} Dye Terminator Cycle Sequencing Ready Reaction Kit from Perkin Elmer, and an Applied Biosystems 373 DNA Sequencer. Homozygous *ptc^{S2}*, *ptc^{tuf2}* mutant embryos, from 10 to 20 hours of development, were selected by lack of fluorescence using the GFP-tagged balancer chromosome *CyO P[w^{+} Kr-GFP]* (Casso et al., 1999).

REFERENCES

Alcedo, J., Ayzenzon, M., Von, O.T., Noll, M. and Hooper, J.E. (1996) The Drosophila smoothened gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. *Cell,* **86**, 221-32.

Alcedo, J., Zou, Y. and Noll, M. (2000) Posttranscriptional regulation of smoothened is part of a self-correcting mechanism in the Hedgehog signaling system. *Mol Cell*, **6**, 457-65.

Blackman, R.K., Sanicola, M., Raftery, L.A., Gillevet, T. and Gelbart, W.M. (1991) An extensive 3' cis-regulatory region directs the imaginal disk expression of decapentaplegic, a member of the TGF-beta family in Drosophila. *Development,* **111**, 657-66.

Blanchette-Mackie, E.J., Dwyer, N.K., Amende, L.M., Kruth, H.S., Butler, J.D., Sokol, J., Comly, M.E., Vanier, M.T., August, J.T., Brady, R.O. and et al. (1988) Type-C Niemann-Pick disease: low density lipoprotein uptake is associated with premature cholesterol accumulation in the Golgi complex and excessive cholesterol storage in lysosomes. *Proc Natl Acad Sci USA*, **85**, 8022-6.

Brand, A.H. and Perrimon, N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development,* **118**, 401-415.

Capdevila, J. and Guerrero, I. (1994) Targeted expression of the signalin molecule Decapentaplegic induces pattern duplications and growth alterations in *Drosophila* wings. *The EMBO Journal,* **13**, 4459-4468.

Capdevila, J., Pariente, F., Sampedro, J., Alonso, J.L. and Guerrero, I. (1994) Subcellular localization of the segment polarity protein patched suggests an interaction with the wingless reception complex in Drosophila embryos. *Development,* **120**, 987-98.

Carstea, E.D., Morris, J.A., Coleman, K.G., Loftus, S.K., Zhang, D., Cummings, C., Gu, J., Rosenfeld, M.A., Pavan, W.J., Krizman, D.B., Nagle, J., Polymeropoulos, M.H., Sturley, S.L., Ioannou, Y.A., Higgins, M.E., Comly, M., Cooney, A., Brown, A., Kaneski, C.R., Blanchette-Mackie, E.J., Dwyer, N.K., Neufeld, E.B., Chang, T.Y., Liscum, L., Tagle, D.A. and et al. (1997) Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis [see comments]. *Science,* **277**, 228-31.

Casso, D., Ramirez-Weber, F.A. and Kornberg, T.B. (1999) GFP-tagged balancer chromosomes for Drosophila melanogaster [corrected and republished in Mech Dev 2000 Mar 1;91(1-2):451-4].*Mech Dev,* **88**, 229-32.

Chen, Y. and Struhl, G. (1996) Dual roles for patched in sequestering and transducing Hedgehog. *Cell,* **87**, 553-63.

Cooper, M.K., Porter, J.A., Young, K.E. and Beachy, P.A. (1998) Teratogen-mediated inhibition of target tissue response to Shh signaling [see comments]. *Science,* **280**, 1603-7.

de Celis, J.F. and Bray, S. (1997) Feed-back mechanisms affecting Notch activation at dorsoventral boundary in the *Drosophila* wing. *development,* **124**, 3241-3251.

Denef, N., Neubuser, D., Perez, L. and Cohen, S.M. (2000) Hedgehog induces opposite changes in turnover and subcellular localization of patched and

smoothened. *Cell,* **102**, 521-31.

Diez del Corral, R., Aroca, P., JL, G.m.-S., Cavodeassi, F. and Modolell, J. (1999) The Iroquois homeodomain proteins are required to specify body wall identity in Drosophila. *Genes Dev,* **13,** 1754-61.

García-Bellido, A., Ripoll, P. and Morata, G. (1973) Developmental compartmentalisation of the wing disk of Drosophila. *Nat New Biol,* **245**, 251-3.

Golic, K.G. (1991) Site-specific recombination between homologous chromosomes in Drosophila. *Science,* **252**, 958-61.

Goodrich, L.V. and Scott, M.P. (1998) Hedgehog and patched in neural development and disease. *Neuron,* **21,** 1243-57.

Hahn, H., Wicking, C., Zaphiropoulous, P.G., Gailani, M.R., Shanley, S., Chidambaram, A., Vorechovsky, I., Holmberg, E., Unden, A.B., Gillies, S., Negus, K., Smyth, I., Pressman, C., Leffell, D.J., Gerrard, B., Goldstein, A.M., Dean, M., Toftgard, R., Chenevix, T.G., Wainwright, B. and Bale, A.E. (1996) Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. *Cell,* **85**, 841-51.

Hooper, J.E. and Scott, M.P. (1989) The Drosophila patched gene encodes a putative membrane protein required for segmental patterning. *Cell,* **59**, 751-65.

Incardona, J.P. and Eaton, S. (2000) Cholesterol in signal transduction. *Curr Opin Cell Biol,* **12**, 193-203.

Incardona, J.P., Gaffield, W., Kapur, R.P. and Roelink, H. (1998) The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction.

Development, **125**, 3553-62.

Ingham, P.W. (1998) Transducing Hedgehog: the story so far. *Embo J,* **17**, 3505-11.

Johnson, R.L., Rothman, A.L., Xie, J., Goodrich, L.V., Bare, J.W., Bonifas, J.M., Quinn, A.G., Myers, R.M., Cox, D.R., Epstein, E.J. and Scott, M.P. (1996) Human homolog of patched, a candidate gene for the basal cell nevus syndrome. *Science,* **272**, 1668-71.

Kobayashi, T., Beuchat, M.H., Lindsay, M., Frías, S., Palmiter, R.D., Sakuraba, H., Parton, R.G. and Gruenberg, J. (1999) Late endosomal membranes rich in lysobisphosphatidic acid regulate cholesterol transport. *Nat Cell Biol;* **1**, 113-118.

Lawrence, P.A. and Struhl, G. (1996) Morphogens, compartments, and pattern: lessons from drosophila? *Cell,* **85**, 951-61.

Loftus, S.K., Morris, J.A., Carstea, E.D., Gu, J.Z., Cummings, C., Brown, A., Ellison, J., Ohno, K., Rosenfeld, M.A., Tagle, D.A., Pentchev, P.G. and Pavan, W.J. (1997) Murine model of Niemann-Pick C disease: mutation in a cholesterol homeostasis gene [see comments]. *Science,* **277**, 232-5.

Marigo, V., Scott, M.P., Johnson, R.L., Goodrich, L.V. and Tabin, C.J. (1996) Conservation in Hedgehog signaling: induction of a chicken patched homolog by Sonic Hedgehog in the developing limb. *Development,* **122**, 1225-1253.

Motzny, C.K. and Holmgren, R. (1995) The Drosophila cubitus interruptus protein and its role in the wingless and hedgehog signal transduction pathways. *Mech Dev,* **52**, 137-150.

Mullor, J.L., Calleja, M., Capdevila, J. and Guerrero, I. (1997) Hedgehog activity, independent of Decapentaplegic, participates in wing disc patterning. *Development,* **124,** 1227-1237.

Mullor, J.L. and Guerrero, I. (2000) A gain-of-function mutant of patched dissects different responses to the Hedgehog gradient. *Dev Biol,* **In press.**

Nakano, Y., Guerrero, I., Hidalgo, A., Taylor, A., Whittle, J.R. and Ingham, P.W. (1989) A protein with several possible membrane-spanning domains encoded by the Drosophila segment polarity gene patched. *Nature,* **341,** 508-13.

Nestoras, K., Lee, H. and Mohler, J. (1997) Role of knot (kn) in wing patterning in Drosophila. *Genetics,* **147,** 1203-12.

Neufeld, E., Wastrey, M., Patel, S., Suresh, S., Cooeney, A., Dwyer, N.K., Roff, C., Ohno, K., Morris, J., Carstea, E. and al., e. (1999) The Niemann-Pick C1 protein resides in a vesicular compartment linked to retrograde transport of multiple lysosomal cargo. *J Biol Chem,* **274,** 9627-9635.

Nohturfft, A., Hua, X., Brown, M.S. and Goldstein, J.L. (1996) Recurrent G-to-A substitution in a single codon of SREBP cleavage-activating protein causes sterol resistance in three mutant Chinese hamster ovary cell lines. *Proc Natl Acad Sci USA;* **93,** 13709-14.

Ohlmeyer, J.T. and Kalderon, D. (1998) Hedgehog stimulates maturation of Cubitus interruptus into a labile transcriptional activator. *Nature,* **396,** 749-53.

Patel, N.H., Martin, B.E., Coleman, K.G., Poole, S.J., Ellis, M.C., Kornberg, T.B. and Goodman, C.S. (1989) Expression of engrailed proteins in arthropods, annelids, and chordates. *Cell,* **58,** 955-68.

Pignoni, F. and Zipursky, S.L. (1997) Induction of Drosophila eye development by decapentaplegic. *Development,* **124,** 271-8.

Ramirez-Weber, F.A., Casso, D.J., Aza-Blanc, P., Tabata, T., Kornberg, T.B., Lin, H., Ruiz, I.A.A., Alcedo, J., Zou, Y. and Noll, M. (2000) Hedgehog signal transduction in the posterior compartment of the *Drosophila* wing imaginal disc. *Mol Cell, 6,* 479-85.

Rietveld, A., Neutz, S., Simons, K. and Eaton, S. (1999) Association of sterol- and glycosylphosphatidylinositol-linked proteins with Drosophila raft lipid microdomains. *J Biol Chem,* **274**, 12049-54.

Sanchez Herrero, E., Couso, J.P., Capdevila, J. and Guerrero, I. (1996) The fu gene discriminates between pathways to control dpp expression in Drosophila imaginal discs. *Mech Dev,* **55**, 159-70.

Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature,* **387**, 569-572.

Tabata, T. and Kornberg, T.B. (1994) Hedgehog is a signaling protein with a key role in patterning Drosophila imaginal discs. *Cell,* **76**, 89-102.

Taipale, J., Chen, J.K., Cooper, M.K., Wang, B., Mann, R.K., Milenkovic, L., Scott, M.P. and Beachy, PA. (2000) Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine [see comments]. *Nature,* **406**, 1005-9.

van den Heuvel, M. and Ingham, P.W. (1996) smoothened encodes a receptor-like serpentine protein required for hedgehog signalling. *Nature,* **382,** 547-51.

Vervoort, M., Crozatier, M., Valle, D. and Vincent, A. (1999) The COE transcription factor collier is a mediator of short-range hedgehog-induced patterning of the drosophila wing. *Curr Biol,* **9**, 632-9.

Xie, J., Murone, M., Luoh, S.M., Ryan, A., Gu, Q., Zhang, C., Bonifas, J.M., Lam, C.W., Hynes, M., Goddard, A., Rosenthal, A., Epstein, E.H., Jr. and de Sauvage, F.J. (1998) Activating Smoothened mutations in sporadic basal-cell carcinoma. *Nature,* **391,** 90-2.

Xu, T. and Rubin, G.M. (1993) Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development,* **117,** 1223-37.

CLAIMS

1. Use of a compound that blocks HH signalling through modifications of PTC and SMO vesicular sorting in the preparation of a medicament for the treatment of a mammalian cancer.

2. Use according to claim 1, wherein the cancer is a skin cancer.

3. Use according to claim 2, wherein the cancer is a basal cell carcinoma.

4. Use according to any of claims 1 to 3, wherein the compound is an analogue or derivative of cholesterol.

5. A method of assaying the ability of a compound to block HH signalling through modifications of PTC and SMO vesicular sorting, comprising:

establishing a mammalian cell culture system in which a reporter gene is placed under the control of a promoter which is dependent on Hedgehog signalling for expression;

treatment of the culture with a test compound;

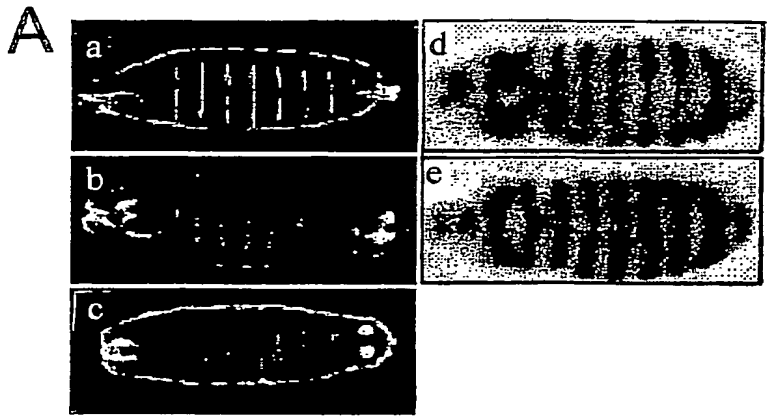
measuring a product of the reporter gene.

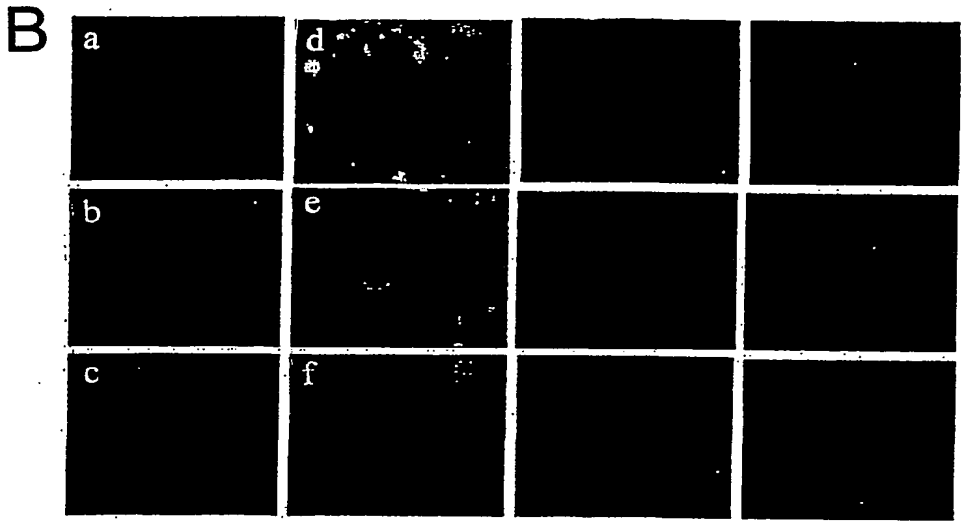
6. A method according to claim 5, wherein the reporter gene is firefly luciferase and the product is chemoluminescence.

7. A compound detected by an assay method according to claim 5 or claim 6.

8. A method of treating a mammal affected by cancer comprising administering to the affected mammal a therapeutically effective amount of a compound that blocks HH signalling through modifications of PTC and SMO vesicular sorting.

9. A pharmaceutical composition comprising as an active ingredient a compound that blocks HH signalling through modifications of PTC and SMO vesicular sorting.





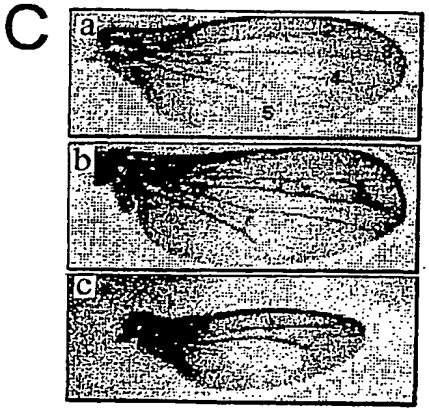


Fig. 1

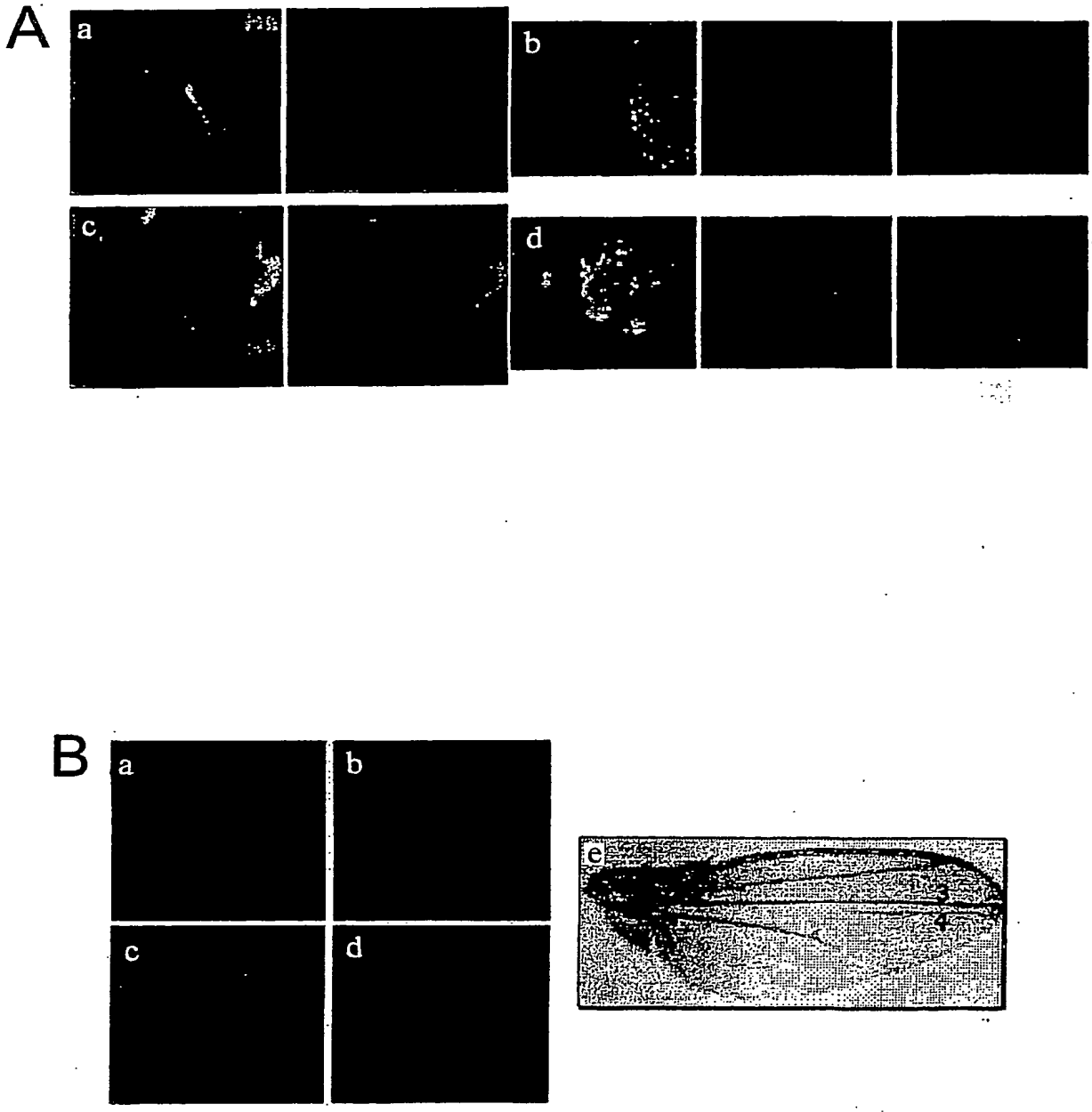


Fig. 2

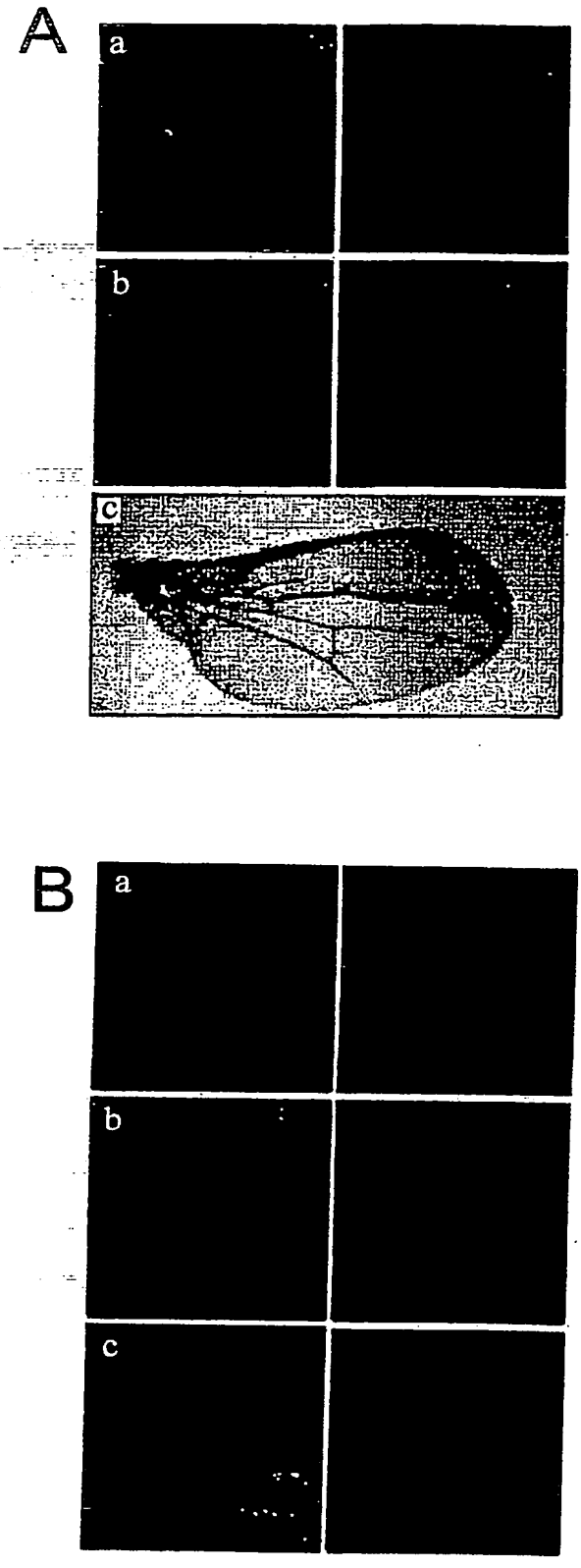


Fig. 3

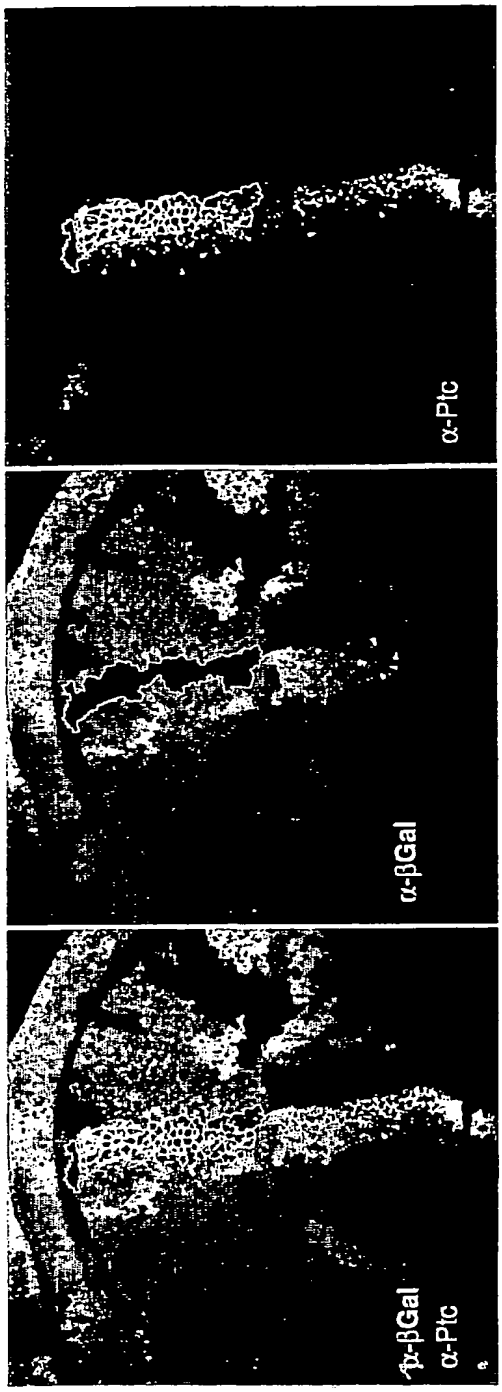


Fig. 4